

## SPECIFICATION

METHOD FOR STABILIZING PROTEIN SOLUTION FORMULATIONSTECHNICAL FIELD

5           The present invention relates to a method for stabilizing protein solution formulations and to a storage container for holding protein solution formulations. More specifically, the present invention relates to a method for stabilizing physiologically active protein formulations by  
10 storing protein solution formulations under magnetic field lines, as well as to a magnetic field generator-equipped storage container for holding protein solution formulations. The method and storage container of the present invention suppress the association of physiologically active proteins  
15 and are particularly useful for stabilizing protein solution formulations.

BACKGROUND ART

Advances in gene recombination technology have  
20 enabled a stable supply of various physiologically active protein formulations. Most of these physiologically active proteins are known to be associated in an aqueous solution, which constitutes a major cause of reducing the stability of these formulations. To ensure the stability of these  
25 formulations, they are provided as lyophilized formulations or in the form of protein solution formulations supplemented with various additives for improving the stability.

For example, it has been found that stabilization effects are obtained by addition of stabilizers including high-molecular weight materials such as proteins (e.g., human serum albumin, purified gelatin) or low-molecular weight materials such as polyols, amino acids and surfactants. However, when added as stabilizers, organism-derived high-molecular weight materials such as proteins are disadvantageous in that very complicated processes are necessary to remove contaminants, such as viruses, originating from the stabilizers per se. Also, when heat treatment is carried out for the purpose of virus inactivation, such stabilizers may cause problems such as association and aggregation under heat stress.

For this reason, there has been a strong demand for a method for stabilizing protein solution formulations, which minimizes the amounts of stabilizers and allows suppression of protein molecule association through simple treatment.

The object of the present invention is to provide a method for improving the stability of protein solution formulations by suppressing the formation of associated matter from molecules such as physiologically active proteins (e.g., antibodies, enzymes, hormones, cytokines) in a solution form.

## 25 DISCLOSURE OF THE INVENTION

As a result of extensive and intensive efforts made to achieve the above object, the inventors of the present invention have unexpectedly found that the formation of

associated matter (e.g., dimers) can be significantly suppressed when protein solution formulations are placed under magnetic field lines. This finding led to the completion of the present invention.

5           Namely, the present invention provides the following:

- (1) a method for stabilizing a protein solution formulation, which comprises storing the protein solution formulation under magnetic field lines;
- (2) the method according to (1) above, wherein the  
10 magnetic flux density is 1 mT (millitesla) or more;
- (3) the method according to (1) or (2) above, wherein the protein is a physiologically active protein;
- (4) the method according to (3) above, wherein the physiologically active protein is selected from an antibody,  
15 an enzyme, a cytokine and a hormone;
- (5) the method according to (4) above, wherein the physiologically active protein is a hematopoietic factor;
- (6) the method according to (5) above, wherein the hematopoietic factor is erythropoietin or granulocyte  
20 colony-stimulating factor;
- (7) the method according to (4) above, wherein the physiologically active protein is an antibody;
- (8) the method according to (1) above, wherein the protein solution formulation is in the form of a pre-filled  
25 syringe formulation;
- (9) a storage container for holding a protein solution formulation, which comprises a magnetic field generator;
- (10) the storage container according to (9) above, wherein

- the magnetic field generator is a magnet;
- (11) a method for stabilizing a protein-containing solution, which comprises storing the protein-containing solution under magnetic field lines;
- 5 (12) the method according to (11) above, wherein the protein-containing solution is a bulk solution for protein production;
- (13) a stabilized protein formulation, which is prepared from a bulk solution for protein production stored under
- 10 magnetic field lines;
- (14) the formulation according to (13) above, wherein the stabilized protein formulation is in the form of a solution formulation;
- (15) the formulation according to (13) above, wherein the
- 15 stabilized protein formulation is in the form of a lyophilized formulation;
- (16) a method for suppressing the formation of associated matter in a protein solution formulation, which comprises storing the protein solution formulation under magnetic
- 20 field lines; and.
- (17) the use of a magnetic field generator for stabilization of a protein solution formulation.

#### BRIEF DESCRIPTION OF DRAWINGS

- 25 Figure 1 shows a schematic view of an accelerated test for injectable EPO formulations under magnetic field lines.

Figure 2 shows an embodiment for storing solution

formulations according to the method of the present invention (a: side view; b: top view).

Figure 3 shows a side view of an embodiment for storing solution formulations according to the method of the present invention.

Figure 4 shows an embodiment for storing G-CSF solutions under magnetic field lines.

#### BEST MODE FOR CARRYING OUT THE INVENTION

Proteins used in the present invention are preferably, but not limited to, physiologically active proteins. The protein solution of the present invention is not limited to pharmaceutical use, and it is therefore intended to provide a method for stabilizing any protein-containing solutions in which protein stabilization is required. Such proteins are contained in pharmaceuticals, foods, cosmetics and the like. Physiologically active proteins include, but are not limited to, antibodies, enzymes, cytokines and hormones. Specific examples include, but are not limited to, hematopoietic factors such as granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), erythropoietin (EPO) and thrombopoietin, cytokines such as interferons, IL-1 and IL-6, immunoglobulins such as monoclonal antibodies and humanized antibodies, tissue plasminogen activator (TPA), urokinase, serum albumin, blood coagulation factor VIII, leptin, insulin, and stem cell growth factor (SCF). Among these physiologically active proteins, preferred are

hematopoietic factors such as EPO, G-CSF, GM-CSF and thrombopoietin, and more preferred are EPO and G-CSF. Also preferred as physiologically active proteins are immunoglobulins, and more preferred are monoclonal  
5 antibodies and humanized antibodies.

The physiologically active protein as used in the stabilization method of the present invention is intended to mean a protein having substantially the same biological activities as a corresponding physiologically active  
10 protein of mammalian (especially human) origin. Such a protein may either be native or genetically recombinant, preferably genetically recombinant. Genetically recombinant and physiologically active proteins may be prepared by production in bacterial cells such as *E. coli*;  
15 yeast cells; or animal-derived cultured cells such as Chinese hamster ovary (CHO) cells, C127 cells or COS cells. The proteins thus prepared are isolated and purified in various manners before use. Such genetically recombinant proteins encompass those having the same amino acid  
20 sequence as the corresponding native protein, as well as those comprising deletion, substitution or addition of one or more amino acids in the amino acid sequence, but retaining the biological activities mentioned above. Further, such proteins include those chemically modified  
25 with PEG, etc.

Examples of a physiologically active protein include glycoproteins having sugar chains. Although the origin of sugar chains is not limited in any way, preferred are sugar

chains attached to mammalian cells. Mammalian cells include, for example, Chinese hamster ovary (CHO) cells, BHK cells, COS cells and human-derived cells, with CHO cells being most preferred.

5           When a physiologically active protein is G-CSF, any G-CSF can be used as long as it is highly purified. G-CSF as used herein may be prepared in any manner; for example, by culturing human tumor cell lines or by genetically engineered production in bacterial cells such as *E. coli*;  
10 yeast cells; or animal-derived cultured cells such as Chinese hamster ovary (CHO) cells, C127 cells or COS cells. G-CSF thus prepared is extracted, isolated and purified in various manners before use. Preferred are those produced by genetic recombination techniques in *E. coli* cells, yeast  
15 cells or CHO cells. The most preferred are those produced recombinantly in CHO cells. In addition, G-CSF may be chemically modified with PEG, etc. (see International Publication No. WO90/12874).

          When a physiologically active protein is EPO, it may  
20 be prepared in any manner. Examples of EPO in the present invention include those derived from human urine as well as those produced in Chinese hamster ovary (CHO) cells, BHK cells, COS cells, human-derived cells or the like (e.g., as described in JP KOKAI 61-12288) by genetic engineering  
25 techniques. EPO thus prepared is extracted, isolated and purified in various manners before use. In addition, EPO may be chemically modified with PEG, etc. (see International Publication No. WO90/12874). EPO as used

herein further includes unglycosylated EPO that is chemically modified with PEG, etc. Likewise, EPO analogs are also included, which are modified to have at least one additional site for N-linked or O-linked glycosylation in the amino acid sequence of EPO (see, e.g., JP KOKAI 08-151398, JP KOHYO 08-506023). Instead of increasing the number of glycosylation sites, EPO analogs may also be modified to have an increased content of sialic acid for attaining additional sugar chains.

When a physiologically active protein is a monoclonal antibody, it may be prepared in any manner. In general, a monoclonal antibody can be produced using known techniques by immunizing a sensitizing antigen in accordance with conventional procedures for immunization, fusing the resulting immunocytes with known parent cells through conventional procedures for cell fusion, and then screening monoclonal antibody-producing cells through conventional procedures for screening.

Alternatively, antibody genes are cloned from hybridomas, integrated into appropriate vectors, and then transformed into hosts to produce antibody molecules using gene recombination technology. The genetically recombinant antibodies thus produced may also be used in the present invention (see, e.g., Carl, A. K. Borrebaeck, James, W. Larrick, THERAPEUTIC MONOCLONAL ANTIBODIES, Published in the United Kingdom by MACMILLAN PUBLISHERS LTD, 1990). More specifically, cDNA of antibody variable domains (V domains) is synthesized from hybridoma mRNA using reverse



transcriptase. Upon obtaining DNA encoding the target antibody V domains, the DNA is ligated to DNA encoding desired antibody constant domains (C domains) and integrated into an expression vector. Alternatively, the  
5 DNA encoding the antibody V domains may be integrated into an expression vector carrying the DNA of the antibody C domains. The DNA construct is integrated into an expression vector such that it is expressed under control of an expression regulatory region, e.g., an enhancer or a  
10 promoter. Host cells are then transformed with this expression vector for antibody expression.

In the present invention, it is possible to use genetically recombinant antibodies (e.g., chimeric antibodies and humanized antibodies) that are artificially  
15 modified with a view to attenuating the characteristics as heteroantigen to human. These modified antibodies may be prepared in a known manner. A chimeric antibody is composed of variable domains of heavy and light chains from a non-human mammalian (e.g., mouse) antibody and constant  
20 domains of heavy and light chains from a human antibody. To obtain chimeric antibodies, DNAs encoding such mouse antibody variable domains may be ligated to DNAs encoding the human antibody constant domains, and then integrated into an expression vector, followed by transformation into  
25 a host for antibody production.

Humanized antibodies are also called reshaped human antibodies and are obtained by grafting complementarity determining regions (CDRs) of non-human mammalian (e.g.,

mouse) antibodies to replace those of human antibodies. Standard gene recombination procedures for this purpose are also known. More specifically, a DNA sequence is designed to allow ligation between CDRs of mouse antibody and  
5 framework regions (FRs) of human antibody, and is synthesized by PCR from several oligonucleotides which are prepared so as to have sections overlapping with one another at the ends. The DNA thus obtained is ligated to DNA encoding human antibody constant domains, and  
10 integrated into an expression vector, followed by transformation into a host for antibody production (see European Patent Publication No. EP 239400 and International Publication No. WO 96/02576). The FRs of human antibody, which is ligated via CDRs, are selected such that the  
15 complementarity determining regions form a favorable antigen-binding site. If necessary, amino acid substitution(s) may be made in the framework regions of antibody variable domains such that the complementarity determining regions of reshaped humanized antibody may form  
20 an appropriate antigen-binding site (Sato, K. et al., Cancer Res. (1993) 53, 851-856).

A humanized anti-IL-6 receptor antibody (hPM-1) can be presented as a preferred example for such reshaped humanized antibodies (see International Publication No.  
25 WO92-19759). In addition, a humanized anti-HM1.24 antigen monoclonal antibody (see International Publication No. WO98-14580), a humanized anti-parathyroid hormone-related peptide antibody (anti-PTHrP antibody; see International

Publication No. WO98-13388), a humanized anti-tissue factor antibody (see International Publication No. WO99-51743) and the like are also preferred for use in the present invention.

5           Procedures for obtaining human antibodies are also known. For example, human lymphocytes are sensitized *in vitro* with a desired antigen or a desired antigen-expressing cell, and the sensitized lymphocytes are then fused with human myeloma cells (e.g., U266) to give desired  
10 human antibodies having binding activity to the antigen (see JP KOKOKU 01-59878). Alternatively, transgenic animals having the entire repertoires of human antibody genes may be immunized with an antigen to obtain desired human antibodies (see International Publication Nos. WO  
15 93/12227, WO 92/03918, WO 94/02602, WO 94/25585, WO 96/34096 and WO 96/33735). There are another techniques using human antibody libraries to give human antibodies by panning. For example, human antibody variable domains may each be expressed by phage display technology as a single-  
20 chain antibody (scFv) on the surface of phages, followed by selection of phages binding to the antigen. When genes of the selected phages are analyzed, it is possible to determine DNA sequences encoding human antibody variable domains binding to the antigen. Once the DNA sequences of  
25 scFv binding to the antigen have been identified, the sequences may be used to construct appropriate expression vectors to obtain human antibodies. These techniques are already well known and can be found in WO 92/01047, WO

92/20791, WO 93/06213, WO 93/11236, WO 93/19172, WO 95/01438 and WO 95/15388.

In another embodiment, human antibodies produced in transgenic animals and the like are also preferred.

5           Furthermore, the antibody as used herein encompasses antibody fragments including Fab, (Fab')<sub>2</sub>, Fc, Fc' and Fd, as well as reshaped antibodies including monovalent or multivalent single chain antibodies (scFV).

10           As used herein, the term "physiologically active protein-containing sample" is intended to mean a sample containing any protein, whether native or recombinant. It is preferably a culture medium of mammalian cells (e.g., CHO cells) containing physiologically active protein molecules produced by culture, which may further be  
15           subjected to partial purification or other certain treatment(s).

          If desired, a physiologically active protein solution formulation may further comprise, in addition to a physiologically active protein, other ingredients such as a  
20           stabilizer (e.g., surfactants, amino acids), a diluent, a solubilizer, an excipient, a pH adjuster, a soothing agent, a buffering agent, a sulfur-containing reducing agent and an antioxidant. Such a formulation may be prepared by dissolving these ingredients in a commonly used buffer.

25           Physiologically active protein solution formulations can usually be provided in containers having a definite volume, including sealed and sterilized plastic or glass vials, ampoules and syringes, as well as in large volume

containers like bottles. The stabilization method of the present invention can be used for solution formulations contained in any of these containers. Preferred are solution formulations in the form of pre-filled syringe  
5 formulations.

In addition, vials are also presented as a preferred example.

In the present invention, solution formulations of physiologically active proteins are stored under magnetic  
10 field lines. Examples of a magnetic field generator include, but are not limited to, a magnet, a magnetic head produced by semiconductor processes, and an array of microcoils through which an electric current is passed. Preferably, containers of the solution formulations are  
15 surrounded by magnets because they are inexpensive and easy to use. Figure 1 shows an embodiment of the present invention. Although such a magnet may be of any shape and may be placed in any orientation, a sheet-shaped magnet is preferred in view of the packaged form of the solution  
20 formulations. Also, taking into account the fact that the intensity of magnetic field lines depends on the distance from the magnet surface as well as the demand that drug solutions in syringes should be distributed as uniformly as possible, a magnet(s) is preferably placed such that the  
25 S-N direction is perpendicular to the longitudinal axis of the syringes (see Figure 2). One or more magnetic field generators are arranged in such a manner that physiologically active protein solution formulations to be

held are placed under magnetic field lines. Syringes may be arranged on one sheet-shaped magnet as shown in Figure 2 or may be arranged between two sheet-shaped magnets as shown in Figure 3.

5           Alternatively, vials may be arranged on a sheet-shaped magnet as shown in Figure 4.

Physiologically active protein solution formulations and a magnet(s) may be placed in any arrangement as long as the solution formulations are stored under magnetic field  
10   lines. The magnetization of the magnet(s) may be oriented in any direction as long as the solution formulations are stored under magnetic field lines. For example, in Figure 1, the magnet may be placed in such a manner that the upper surface is N pole and the lower surface is S pole, or vice  
15   versa. In Figures 2 and 4, the surface contacted with syringes or vials may be either N pole or S pole. In Figure 3, one of two surfaces contacted with syringes may be N pole and the other S pole, or alternatively both of them may be the same pole.

20           The magnetic flux density (i.e., the intensity of magnetic field) is 1 mT or more, preferably 1 to 450 mT, and more preferably 1 to 150 mT.

The present invention further provides a storage container for holding physiologically active protein  
25   solution formulations, which is equipped with a magnetic field generator. Although the storage container of the present invention may be of any shape and may be of any material, it has a site(s) for holding the above-mentioned

magnetic field generator(s) and a solution formulation(s). The storage container may be a storage box, a refrigerator, a transport container or the like.

The present invention further provides a method for  
5 stabilizing a protein bulk solution, which comprises  
storing the protein bulk solution under magnetic field  
lines. The present invention also enables the preparation  
of stabilized protein formulations from a protein bulk  
solution stored under magnetic field lines. Such  
10 stabilized protein formulations may be either in the form  
of solution formulations or lyophilized formulations.

When stored according to the method of the present  
invention, physiologically active protein solution  
formulations have been found to suppress the formation of  
15 associated matter even after long-term storage. For  
example, after injectable erythropoietin (EPO) formulations  
(750 IU) were subjected to an accelerated test in condition  
that they were stored at 40°C for 2 weeks and 1 month in  
the absence of magnetic field lines, the formulations were  
20 evaluated by purity test (SDS-PAGE) and quantification test  
(liquid chromatography). As a result, they were found to  
show increased amounts of dimers in the purity test and  
reduced levels of EPO content in the quantification test.  
For comparison, the same purity test (SDS-PAGE) and  
25 quantification test (liquid chromatography) were performed  
on injectable EPO formulations (750 IU) after they were  
subjected to an accelerated test in the condition that they  
stored at 40°C for 2 weeks and 1 month under magnetic field

lines (about 100 mT). The results indicate that there was no change in any property to be evaluated when compared to unaccelerated formulations, except that the formulations accelerated at 40°C for 1 month showed slightly increased  
5 amounts of dimers in the purity test.

Without being bound by any particular theory, the inventors of the present invention estimate the mechanism of the present invention as follows. Namely, it is generally believed that protein association may be caused  
10 by binding between hydrophobic moieties of protein molecules, which are exposed at the surface of their three-dimensional structure due to some influence (e.g., heat or collisions with the container surface). Thus, if protein molecules in an aqueous solution can be restrained by some  
15 force, random collisions of protein molecules can be prevented. In the present invention, it is believed that protein molecules in an aqueous solution are arranged regularly under a strong magnetic field and hence are restrained to prevent their random collisions, thus  
20 suppressing their association caused by binding between hydrophobic moieties of the molecules.

The method and storage container of the present invention are therefore based on a technical idea completely different from the conventional approach in  
25 which stabilizers are added to stabilize physiologically active protein formulations. The present invention suppresses the formation of associated matter through a simple process and also enables long-term storage of the



formulations. The method and the storage container of the present invention can be used to reduce the amount of additives to be used.

5 The present invention will now be further described in the following examples, which are not intended to limit the scope of the invention. Based on the detailed description, various changes and modifications will be apparent to those skilled in the art, and such changes and modifications fall within the scope of the invention.

10

#### EXAMPLES

##### Example 1: Effects in EPO formulations

###### 1) Samples

Injectable EPO formulations (750 IU) were used  
15 (syringe formulations, EPO concentration: 1500 IU/ml, volume: 0.5 ml, Chugai Pharmaceutical Co., Ltd.).

###### 2) Test procedures

Injectable EPO formulations (750 IU) were provided  
20 with a magnet (about 100 mT) (see Figure 1) and stored in a thermostatic chamber (40°C) for 2 weeks and 1 month before being evaluated. In Figure 1, the sheet-shaped magnet was placed in such a manner that the upper surface was N pole and the lower S pole.

25 Simultaneously, samples were subjected to an accelerated test in the absence of magnetic field lines (<1 mT) at 40°C for 2 weeks and 1 month. The samples accelerated as above as well as unaccelerated samples were

provided for evaluation. Each sample was evaluated by changes in EPO content, dimer content and other relative substance content as compared to the unaccelerated samples.

5 3) Evaluation procedures

3-1) Purity test (SDS-gel electrophoresis)

The samples prepared above are each used as a sample solution. Exactly 60  $\mu$ L of each sample solution is taken and supplemented with non-reducing Sample Buffer<sup>\*1</sup> (exactly  
10 20  $\mu$ L), followed by heating in a warm bath (50°C) for 15 minutes. After heating, BPB Solution<sup>\*2</sup> (exactly 4.0  $\mu$ L) is added to give an electrophoresis sample.

The electrophoresis samples and a molecular weight marker<sup>\*3</sup> (7.5  $\mu$ L each) are tested by electrophoresis under  
15 the following conditions and detected by Western blotting.

3-1-1) Electrophoresis

Apparatus: TEFCO electrophoresis unit

Gel: slab gel [gradient: 8% to 16%, 15 wells, 1.5 mm  
20 thickness, TEFCO]

Condition: constant current at 25 mA/gel

Duration: about 1.5 hours

Electrophoresis buffer: Electrophoresis Buffer<sup>\*4</sup>

25 3-1-2) Western blotting

After electrophoresis, the gel is soaked in Transfer Buffer<sup>\*5</sup> (10 ml) and equilibrated for 30 minutes. Next, a sponge and a sheet of filter paper (first transfer filter

paper, 20 cm x 10 cm, Pharmacia), both of which have been soaked in Transfer Buffer, are placed in sequence on the anode (+) side of a transfer unit and stacked with a ProBlot membrane<sup>\*6</sup>. The ProBlot membrane has been soaked in  
5 methanol for about 30 seconds and further in Transfer Buffer for 5 minutes in advance. The equilibrated gel is placed on top of the membrane and further stacked with one sheet of filter paper and a sponge, both of which have been soaked in Transfer Buffer. The stack thus obtained is  
10 placed such that the membrane side faces the anode (+) and the gel side faces the cathode (-). The gel is transferred to the ProBlot membrane under the following conditions, followed by color development of the ProBlot membrane using anti-EPO rabbit serum.

15        Temperature: constant about 25°C  
         Condition: constant voltage at  $65 \pm 2$  V  
         Duration: 120 minutes

         \*1 Non-reducing Sample Buffer: Disodium  
20 ethylenediaminetetraacetate (74.48 mg) and sodium lauryl sulfate (5.0 g) are dissolved in Tris-HCl Solution<sup>\*7</sup> (50 ml).

         \*2 BPB Solution: Tris-HCl Solution (0.625 ml),  
concentrated glycerin (3.5 ml) and bromphenol blue solution  
(5 mg) are mixed. The resulting solution is cold stored at  
25 4°C.

         \*3 Molecular weight marker solution: Prestained SDS-PAGE standard solution (Bio-Rad) or an equivalent thereof [containing Lysozyme (molecular weight: 20900), Soybean

trypsin inhibitor (molecular weight: 29100), Carbonic anhydrase (molecular weight: 35500), Ovalbumin (molecular weight: 50600), Bovine serum albumin (molecular weight: 83000) and Phosphorylase B (molecular weight: 101000)]

5       \*4 Electrophoresis Buffer: Trishydroxymethylamino-methane (3.0 g) and glycine (14.4 g) are added to water (600 ml) and supplemented with sodium lauryl sulfate (1.0 g), followed by addition of water to 1000 ml.

      \*5 Transfer Buffer: Trishydroxymethylaminomethane  
10 (6.0 g) and glycine (28.8 g) are added to water (600 ml) and supplemented with methanol (400 ml), followed by addition of water to 2000 ml.

      \*6 ProBlot membrane: polyvinylidene difluoride membrane filter (8 cm × 8 cm, Applied Biosystems) or an  
15 equivalent thereof

      \*7 Tris-HCl Solution: Trishydroxymethylaminomethane (3.0 g) is dissolved in water (30 ml) and adjusted to pH 6.8 with 1 mol/L hydrochloric acid, followed by addition of water to 100 ml. The resulting solution is cold stored at  
20 4°C.

### 3-2) Quantification (liquid chromatography)

The samples prepared above are each used as a sample solution. A standard preparation of EPO is diluted to 1500  
25 IU/ml with a 0.05% aqueous polysorbate 20 solution for use as a standard solution.

Exactly 100 µL each of each sample solution and the standard solution are tested by liquid chromatography under

the following conditions for measurement of the peak areas of EPO ( $A_T$  and  $A_S$ ), respectively. The following equation is used to determine the EPO content (concentration, mg/ml) for each sample.

5

EPO content (mg/ml)

= an amount of EPO (as EPO polypeptides) in EPO

$$\text{standard preparation (mg/ml)} \times \frac{A_T}{A_S}$$

$A_T$ : EPO peak area of sample solution

10  $A_S$ : EPO peak area of standard solution

#### Operating conditions for liquid chromatography

Detector: UV absorptiometer (detection wavelength: 214 nm)

15 Column: a stainless steel tube (internal diameter: 5 mm, length: 25 cm) filled with about 5  $\mu$ m butylsilylated silica gel (VYDAC 214TP54, Separations Group)

Column temperature: room temperature

Mobile phase: linear gradient of the following two  
20 solutions:

Solution A: a mixture of

water/acetonitrile/trifluoroacetic  
acid (400:100:1)

Solution B: a mixture of

25 acetonitrile/water/trifluoroacetic  
acid (400:100:1)

Gradient operation: The column is equilibrated with

35% Solution B and then injected with a sample solution or the standard solution. After maintaining 35% Solution B for 5 minutes, a linear gradient is run to 100% Solution B over 15 minutes. Then, 100% Solution B is maintained for 2 minutes.

Flow rate: adjusted such that the retention time of EPO is about 18 minutes.

#### 4) Results

##### 4-1) Purity test (SDS-PAGE)

The injectable EPO formulations (750 IU), which were subjected to an accelerated test under magnetic field lines at 40°C for 2 weeks and 1 month, were evaluated by SDS-PAGE.

As a result, the formulation accelerated in the absence of magnetic field lines at 40°C for 2 weeks was found to show an increased amount of dimers, whereas the formulation accelerated under magnetic field lines at 40°C for 2 weeks showed no change when compared to the unaccelerated formulation.

The formulations accelerated at 40°C for 1 month were found to show slightly increased amounts of dimers in both the presence and absence of magnetic field lines, but it was difficult to make a visual comparison of differences in the amount of dimers.

25

##### 4-2) Quantification (liquid chromatography)

The injectable EPO formulations (750 IU), which were subjected to an accelerated test under magnetic field lines

at 40°C for 2 weeks and 1 month, were evaluated by liquid chromatography. The results obtained are shown in Tables 1 and 2.

When compared to the unaccelerated formulation, the  
5 formulation accelerated in the absence of magnetic field lines at 40°C for 2 weeks was found to show a reduced level of EPO content, resulting in a residue level of 96%. Likewise, the formulation accelerated in the absence of magnetic field lines at 40°C for 1 month was found to show  
10 a significantly reduced level of EPO content, resulting in a residue level of 93%.

In contrast, the formulation accelerated under magnetic field lines at 40°C for 2 weeks showed almost no change in EPO content, and also the formulation accelerated  
15 at 40°C for 1 month was found to have a residue level of 97%. When taking into account the precision of this test (repeatability C.V. = 1.6%), there was no change in EPO content in either acceleration period.

Table 1 Residue levels of EPO in formulations accelerated at 40°C for 2 weeks

	Magnetic flux density	EPO conc. (of label)	Residue (%)
Unaccelerated	<1 mT	93.8%	100.0%
40°C for 2 weeks	<1 mT	89.7%	95.6%
40°C for 2 weeks	100 mT	93.7%	99.9%

Table 2 Residue levels of epoetin beta in formulations accelerated at 40°C for 1 month

	Magnetic flux density	EPO conc. (of label)	Residue (%)
Unaccelerated	<1 mT	93.9%	100.0%
40°C for 1 month	<1 mT	87.5%	93.1%
40°C for 1 month	100 mT	91.4%	97.3%

## 5 Example 2: Effects in G-CSF solutions

### 1) Sample preparation

A G-CSF solution (G-CSF concentration: about 0.5 mg/ml) was filtered through a disposable filter and filled into 5 ml glass vials in an amount of 1 ml per vial for use as test samples.

### 2) Storage conditions

These test samples were stored in a thermostatic chamber (30°C) in both the presence of magnetic field lines (about 100 mT<sup>\*8</sup>) and the absence of magnetic field lines<sup>\*9</sup> (see Figure 4). In Figure 4, the sheet-shaped magnet was placed in such a manner that the surface contacted with the vials was N pole and the other S pole.

\*8 Tesla (T): the unit of magnetic flux density,



1 tesla = 10,000 gauss (G)

\*9 In the absence of magnetic field lines: magnetic flux density found in nature (usually less than 1 mT)

5 3) Evaluation procedures

After being stored in a thermostatic chamber (30°C) for 2 weeks, the test samples were measured for the content of associated G-CSF by gel filtration chromatography (n=3).

10 4) Results

Table 3 shows the content of associated G-CSF after storage at 30°C for 2 weeks.

Table 3 Effects of magnetic field lines on formation behavior of associated G-CSF

Storage conditions	Content of associated G-CSF	
	Average	Standard deviation
In the presence of magnetic field lines	1.5%	0.04%
In the absence of magnetic field lines	2.3%	0.06%

15

As shown in the results above, the magnetic field lines were found to have an effect of suppressing the formation of associated matter during storage of G-CSF solutions.